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Research Article

Ethyl acetate fraction from *Passiflora foetida* promotes rat femoral fracture healing by the BMP-2 signaling pathway

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ABSTRACT

Aim: To evaluate the effect of ethyl acetate fraction (EAF) from *Passiflora foetida* on bone regeneration following bone and marrow injury.

Materials and methods: EAF was administered for two weeks at 50, 100 and 200mg/kg doses orally to adult female *Sprague-Dawley* rats having a drill-hole injury in femur. Analysis of calcein labelling, BMD, micro-architectural parameters, tissue morphology at the drill-hole site, and serum level of bone turnover markers, mineralized nodule formation, expression of osteogenic genes and localization of BMP-2 protein was performed.

Results: EAF dose-dependently accelerated bone regeneration mainly by increasing BMD at the fracture site, serum levels of PINP, OCN, and also mineralized nodule formation in BMCs. In addition, EAF also enhanced microarchitecture of the regenerating bone evident from increased bone volume fraction, trabecular thickness, trabecular number, connective density and decreased trabecular separation and degree of anisotropy. The mechanism studies, EAF accelerated fracture healing in rats by the recruitment of osteoblasts through up-regulation of the BMP-2 signaling pathway at the fracture site.

Conclusion: EAF accelerated fracture healing in rats by the recruitment of osteoblasts through up-regulation of the BMP-2 signaling pathway at the fracture site, therefore could be taken as an alternative therapy for fracture healing.

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Introduction

Bone fractures, one of the most common orthopaedic diseases, eventuate due to accidents or pathological conditions such as osteoporosis [1]. Being more and more common, the expenses of musculoskeletal injuries involved cause significant burdens on public health planning [2]. Repairing of bone at the fracture site is a complex physiological process, commencing after the local bleeding and inflammation, which is accompanied by the complicated activities of mesenchymal precursor cells leading to the formation of soft extracellular matrix tissue, cartilage and the bone [3]. It involves a well-orchestrated pattern of events which is responsible for the migration, proliferation and differentiation of osteoprogenitor cells to endothelial cells and osteoblast cells, which form vascular tissues and bone tissues, respectively, at the fracture site [3].

Metabolic bone disorders involving primary and secondary osteoporosis fundamentally occur due to the reduction in osteoblast function, which leads to high risk of fragility and fracture [4, 5]. A few number of pharmacological interventions which minimize the risk of fractures in osteoporosis contain bisphosphonates, selective estrogen receptor modulators (SERMs) and calcitonin [6-8]. Parathyroid hormone (PTH 1-34) treatment is the only anabolic therapy available in the market for postmenopausal osteoporosis but it has a black-box warning issued by the US FDA because its long term use increases the risk of osteosarcoma in rats [9]. Although these pharmacological interventions are available for clinical use, yet the research is ongoing due to the lack of sufficient benefit-to-risk ratio.

Since there is non-availability of pharmacological interventions as oral administration for rapid fracture repair but traditional mode of plant-based medicine globally has huge mention of herbal extracts which shows positive effect on fracture repair [10]. However, the action of making these effects acceptable via controlled studies is inadequate in the literature. Medicinal plants are the only source of health care management for a huge part of the world's population and continue to play an exuberant role in the health delivery systems. Most of the people in several developing countries still utilize medicinal plants to treat bone related disorders due to the side effects of synthetic drugs and their higher cost. The efficacy of the few medicinal plants extracts to enhance the bone fracture repair process has been reported including *Dalbergia sissoo*, *Spinacia oleracea* and *Peperomia pellucida* [10-12].

Passiflora foetida, commonly known as stinking passion flower, an Indian medicinal plant from the family of Passifloraceae is a herbaceous climber, native of tropical America and found in many parts of India [13]. Traditionally it is used for diarrhoea, intestinal tract, throat, ear infections, fever, skin diseases, vomiting, eczema, chronic ulcer, asthma, biliousness and nervous disorders [14]. It is also reported for *Passiflora foetida* to have sedative, hypnotic, antispasmodic, hepatoprotective and anodyne properties [15]. Recently, we have reported the anti-osteoporotic activity of butanolic fraction from *Passiflora foetida* in ovariectomy-induced bone loss in mice [15]. To the best of our knowledge, fracture healing effect of *Passiflora foetida* has not been scientifically evaluated. Therefore, we demonstrated the effects of ethyl acetate fraction (EAF) from *Passiflora foetida* on fracture healing in rats

using a drill-hole injury model of bone and bone marrow. In this study, fracture healing potential of EAF was investigated in rats using a drill-hole injury model of bone.

Materials and methods

Reagents and chemicals

All chemicals, cell culture media, supplements and PTH were purchased from Invitrogen (Carlsbad, CA), Sigma-Aldrich (St Louis, MO) and Calbiochem (San Diego, CA).

Plant material

Ariel parts of *Passiflora foetida* (L.) were collected from Telangana, India in January 2012, identified and authenticated by a botanist, Dr. N. Venkateshwrlu. A voucher specimen has been preserved in the investigator's laboratory with specimen number CDRI-25012.

Animal study

All the animal studies were designed and approved by the Institutional Animal Ethics Committee (IAEC), Council of Scientific and Industrial Research-Central Drug Research Institute (CSIR-CDRI) and performed according to the regulations of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. Fifty female SD rats (200±20 g) were taken from the National Laboratory Animal Centre of CSIR-CDRI, Lucknow India. Animals were divided into five groups of equal number as follows: control + vehicle (gum acacia in distilled water), EAF (50, 100 and 200 mg/kg/day) and PTH. Treatment to the PTH group was based on our previous published study on SD rats [12]. Animals were kept in a 12h light-dark cycle, with controlled temperature (22–24°C) and humidity (50–60%) and free access to standard rodent food and water.

Drill-hole injury in femur

A drill-hole injury was done in vehicle as well as treatment groups as described earlier [11, 12]. The front skin of the mid portion of the femur in rats was cut straight and longitudinally at 1 cm in length under anesthesia. Femoral bone surface was exposed by stripping the periosteum after splitting the muscle. 2 cm above the knee joint, a drill-hole injury was created with a drill bit of 0.8 mm diameter in the diaphysis of femur. The treatment was started after one day of drill-hole injury and continued for two weeks. One day before autopsy, all animals received intraperitoneal injection of a fluorochrome calcein (20 mg/kg). After the treatment period of the various groups described earlier, all the animals were euthanized and autopsied to collect the femur bones for micro-CT analysis and dynamic histomorphometric study.

Bone mineral density (BMD)

Analysis of the volumetric bone mineral density (vBMD) at the fracture site was conducted by using micro-CT scan following the previously published protocols [16, 17]. Machine was calibrated by using 2 mm diameter hydroxyapatite (HA) phantom rods with known BMD (0.25

g/cm³ and 0.75 g/cm³) [16]. Analysis was done based on a linear correlation between BMD and micro-CT attenuation coefficient [16].

Micro-computed tomography (μCT)

2-D and 3-D analysis of bone internal microstructure of the mineralized tissue at the drill-hole site was performed by using μCT Sky Scan 1076 CT scanner (Aartselaar, Belgium). Bones were scanned with X-ray source of 70KV, 100mA with a pixel size of 18μm. Reconstruction of images was done by using Sky Scan Nrecon software. Network-distributed reconstruction was done with the help of Nrecon software in four personal computers running simultaneously. Bone callus formation area at the fracture site was selected with CT analyzer software by outlining ellipsoid contour. 3-D micro-architecture parameters of bone such as BV/TV (bone volume fraction, %), Tb.Th (trabecular thickness, mm), Tb.Sp (trabecular separation, mm), Tb.N (trabecular number, 1/mm), DA (degree of anisotropy) and Conn.Dn (connection density, 1/mm³) were analysed as described earlier [11, 12].

Bone strength testing

To assess the bone biomechanical properties, we performed the three-point bending test using a bone strength tester (model TK-252C; Muromachi Kikai, Co. Ltd., Tokyo Japan). Femur bones were kept horizontally on the fixture (14 mm span). Vertical rounded point was employed to load on fracture site (drill-hole site). The force displacement curve was noted during loading at the drill-hole site. The maximum force required to break the bone, energy and the stiffness was documented.

Ex-vivo culture of BMCs (Bone marrow cells)

For mineralization study, we followed our previous published study [18]. Bone marrow cells were collected from femur bone and cultured in osteogenic differentiation medium containing 10⁻⁷M dexamethasone. Cells were plated 12-well plates and cultured for 21 days. The medium was refreshed once every 48h. Alizarin Red S dye was used to stain the mineralized nodule formation and quantified by taking optical density at 405nm.

Real-time polymerase chain reaction (qPCR)

Gene expression analysis of BMP-2, Col-I and OCN was performed at the fracture site after EAF treatment for 2 weeks. The drill-hole region of bones including marrow tissue were carefully cut with 2 mm margins with the help of a surgical scalpel, and then crushed in liquid nitrogen [11]. Total RNA was isolated by using TRIzol (Invitrogen) according to the procedure describe by the manufacturer. The concentration of RNA samples was determined by using a spectrophotometer (NANO-Drop). Synthesis of cDNA was done with RevertAid first strand cDNA synthesis kit (Fermentas, Austin, USA) from 2μg of total RNA. SYBR green chemistry was done for quantitative determination of gene expression and the housekeeping gene GAPDH [19, 20]. Primer sequences of the genes used in this study are shown in (Table 1).

Bone turnover markers

Serum bone formation markers such as PINP (procollagen type I N-terminal propeptide) and OCN (osteocalcin) levels were measured after

EAF treatment in the fracture healing progress. During autopsy, blood samples were taken from all the animals followed by serum collection by centrifuging blood samples at 2000g for 20 min at 4°C and stored at -80°C for further analysis [21]. The serum PINP and OCN levels were determined using enzyme-linked immunosorbent assay kit (Qayee Bio-Technology Co. Ltd., Shanghai, China) by following the manufacturer's protocols.

Table 1: Primer sequence of various genes used for qPCR.

Gene Symbol	Gene Name	Primer Sequence	Accession Number
<i>GAPDH</i>	glyceraldehyde-3-phosphate dehydrogenase	F- CAGCAAGGATA CTGAGAGCAAG AG R- GGATGGAATTG TGAGGGAGATG	NM-017008
<i>BMP-2</i>	Bone morphogenetic protein-2	F- CCCCTATATGCT CGACCTGT R- AAAGTTCCTCG ATGGCTTCTT	NM_017178.1
<i>COL1</i>	Type I Collagen	F- CATGTTTCAGCTT TGTGGACCT R- GCAGCTGACTT CAGGGATGT	NM_053304
<i>OCN</i>	Osteocalcin	F- ATAGACTCCGG CGCTACCTC R- CCAGGGGATCT GGGTAGG	NM-013414

qPCR; quantitative polymerase chain reaction, **F;** forward, **R;** reverse

Histological staining

Morphology of the bone tissue at the fracture site was observed after EAF treatment in the fracture healing progress. During autopsy, femur bones with drill-hole injury were dissected out and cleaned. Bones were decalcified in 1% EDTA and subsequently embedded in paraffin. For Histological analysis of the bone at the fracture site, sections of 5μm size were cut through the long axis of femur. Paraffin sections were de-waxed, re-hydrated and stained with haematoxylin and eosin (H&E) [21]. Representative images of the H&E stained sections were selected for observation.

Immunofluorescence

BMP-2 protein expression at the fracture site after EAF treatment was measured by using immunofluorescence method [19, 22, 23]. Femur bones were separated from the knee joint followed by cleaning of soft

tissues. Femur bones with drill-hole sites were fixed in 4% paraformaldehyde, followed by decalcification in 1% EDTA. Then, bones were embedded in paraffin wax to cut 5µm sections. Immunofluorescence analysis of the localization of BMP-2 protein in femoral bone sections at the injury site was performed by using specific antibodies of BMP-2. Deparaffinization and rehydration of the sections were carried out using xylene and ethanol gradient, respectively and permeabilized with 0.1% Triton X-100, then followed by blocking with 1% BSA. Sections were incubated with BMP-2 antibody diluted in 0.5% BSA (1:500) at 4°C for overnight. Then, sections were washed in PBS (pH 7.4), and incubated with Cy-3 goat anti-rabbit antibody (1:300) for 2h at room temperature. Counter staining was done with DAPI for 15 minutes, followed by washing in PBS and mounting with antifade mounting media (Life technologies, Carlsbad, CA, USA). Visualization of the sections was done using Cell Imaging Station (Life technologies, Carlsbad, CA, USA).

Antioxidant activity

DPPH free radical scavenging activity assay

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate), a free radical dye is used to evaluate the antioxidant properties of the plant materials. In this assay, DPPH produces free radicals that gives a violet colour solution and this violet solution is reduced in the presence of an antioxidant molecule, changes measured in spectrophotometric measurement. DPPH was dissolved in methanol, and EAF from *Passiflora foetida* was serially diluted from 1000 µg/ml to 1.95 µg/ml. DPPH radicals and different concentrations of EAF were used in 1:1 ratio. The DPPH solution with or without EAF concentrations was allowed to keep at room temperature for 30 minutes, then measured at 517nm. The DPPH scavenging effect (anti-oxidant activity) of EAF was measured as follows: DPPH scavenging effect (%) = $(\text{Abs} - \text{Abs}_0) / \text{Abs}_0 \times 100$. (Abs is value of DPPH without the sample (EAF); Abs₀ is value of DPPH with EAF concentrations from 1.95 µg/ml to 1000 µg/ml [24]).

NO (Nitric Oxide) free radical scavenging activity assay

Sodium Nitroprusside is a nitric oxide donor. To check the nitric oxide free radical scavenging activity, EAF was serially diluted from 1000 µg/ml to 1.95 µg/ml. 1ml volume was taken from each concentration and 2.0 mL of sodium nitroprusside (10mM) was added in each tube. All samples with or without EAF were incubated for 150 minutes. After the incubation of all samples, 5.0 mL of Griess reagent was added to each sample and the absorbance of chromophore was measured at 546nm. The percentage scavenging activity was calculated: % Scavenging = $[(\text{Abs}_0 - \text{Abs}) / \text{Abs}_0] \times 100$, where Abs₀ is absorbance of control and Abs for EAF sample [25].

Statistical analysis

Data obtained from this study are represented as the mean ± SEM. Analysis of all the data obtained in the experiments with multiple treatments was done by using one-way ANOVA followed by the Newman-Keuls test of significance with the help of GraphPad Prism version 5.0 software. Qualitative observations were represented following assessments made by three individuals blinded to the

experimental designs. Probability values of $p < 0.05$ were considered to be statistically significant.

Results

Effect of EAF on bone regeneration

Quantitative analysis of bone regeneration by using calcein label (mineral deposition) at the drill-hole site was done to check the effect of EAF from *Passiflora foetida*. Oral administration of EAF for 2 weeks enhanced significant mineral deposition than control group. Figure 1A-1C shows representative 2D, 3D drill-hole and 3D callus images of femur bones including drill-hole site by µCT. Figure 1D shows confocal microscopy images of calcein deposition at the same site in different experimental groups. The increase in calcein intensity, compared to the control, was ~48.23% (at 50 mg/kg/day), ~79.25% (at 100 mg/kg/day) and ~69.03% (at 200 mg/kg/day) and was comparable to PTH (Fig. 1E).

Effect of EAF on microarchitecture of regenerated bone at the drill-hole site

After oral administration of EAF for 2 weeks, µCT analysis of the various experimental groups at the drill-hole site was executed. Quantification of micro-architectural parameters of bone revealed that, EAF treatment at a dose of 200 mg/kg/day significantly enhanced the bone micro-architecture by increasing BV/TV (~28.02%), Tb.Th (~27.5%), Tb.N (~22.9%), Conn.Dn (~81.7%) and by decreasing Tb.Sp (~32.22%) and DA (~17.73%), compared to the control group and these results were comparable with PTH (Fig. 2A-2F). Furthermore, two other doses of EAF including 50 and 100mg/kg/day significantly enhanced the bone micro-architectural parameters in the same manner except Tb.Th (Fig. 2B).

Effect of EAF on BMD and bone strength

Volumetric bone mineral density (vBMD) measurement results at the drill-hole site of the femur bones from the different experimental groups were calculated (Fig. 3A). µCT analysis was performed on excised bones of different experimental groups to compare the volumetric BMD. When vBMD of the control group was compared with the results from the EAF (50, 100 and 200 mg/kg/day) treatment groups, a significant decrease by ~30.83%, ~34.16% and ~49.34% could be noticed in the control group, respectively (Fig. 3A). Furthermore, PTH group showed significantly higher vBMD values by ~61.38%, compared to the control group (Fig. 3A). Bone biomechanical strength was also assessed at the drill-hole site, and parameters like power, energy and stiffness were evaluated (Fig. 3B-D). It was noticed that EAF treatment revealed increased power (EAF-50, 100 and 200 mg/kg/day by ~19.45%, ~28.64% and ~31.12%), energy (EAF-50, 100 and 200 mg/kg/day by ~23.32%, ~35.29% and ~40.97%) and stiffness (EAF-50, 100 and 200 mg/kg/day by ~18.19%, ~23.46% and ~37.11%) at the drill site in a dose dependent manner, compared to control. The increase noticed in energy at 200 mg/kg/day dose was comparable to PTH (Fig. 3C).

Effect of EAF on mineralized nodule formation in BMCs

Bone marrow cells (BMCs) from the femurs of all experimental groups were harvested and cultured to induce mineralized nodules formation [26, 27]. Upper panel of figure 4A shows the representative

photomicrograph of alizarin red-S stained cells to show the formation of mineralized nodules. Lower panel of figure 4A represents the quantification of alizarin red-S stained cells. Data from this study showed that EAF and PTH treatment exhibited increase in mineralized nodules formation over control (Fig. 4A). The maximum increase in

mineralization was ~79.91% for 200 mg/kg/day dose followed by ~47.26% and ~58.42% for 50 and 100 mg/kg/day doses compared to the control group. PTH the positive control showed the best response by increasing the nodules formation by ~105.73%.

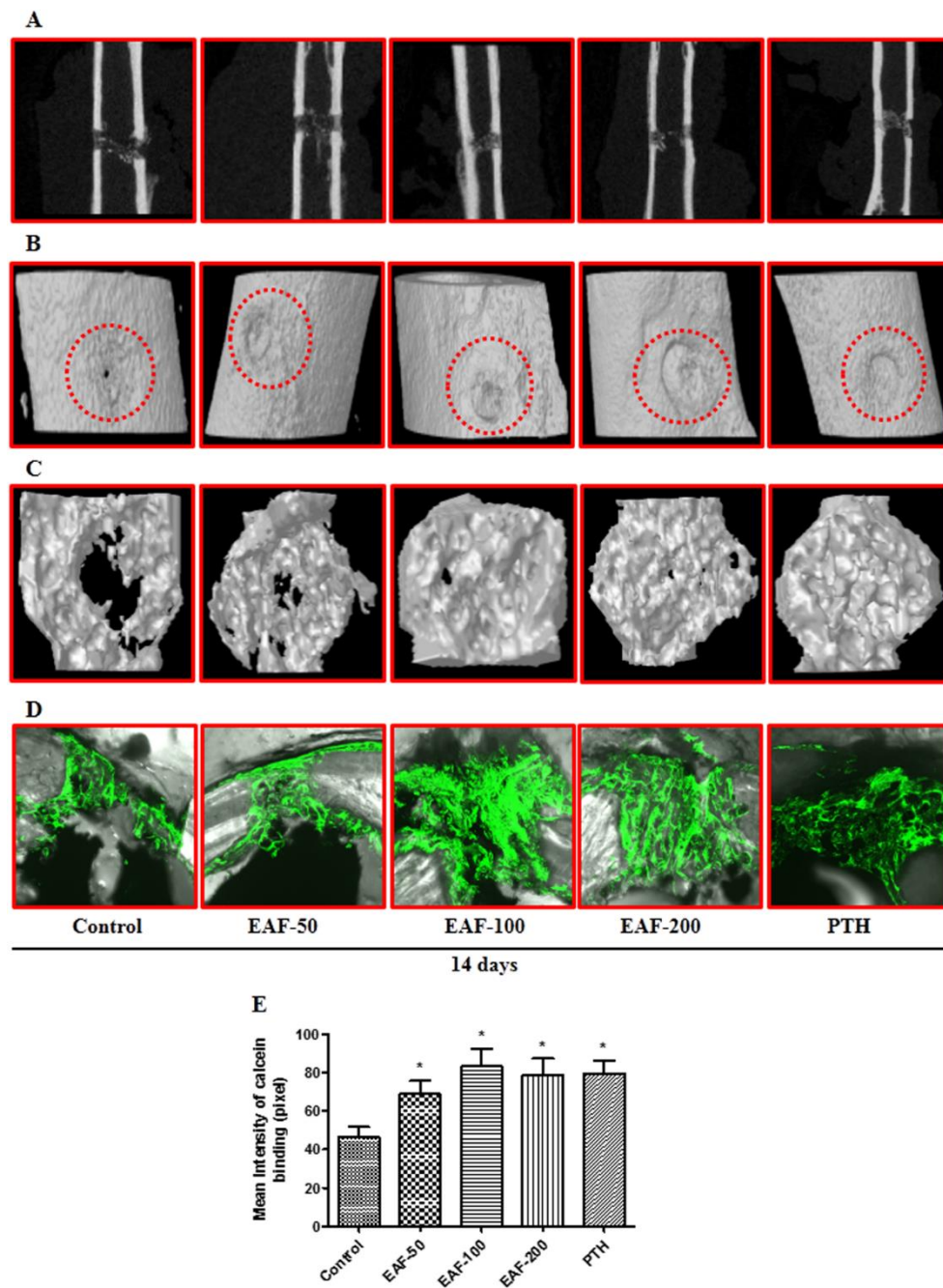


Fig 1: EAF treatment promoted bone regeneration in the drill-hole (fracture site). Representative (A) 2D, (B) 3D drill-hole and (C) 3D callus images by μ -CT from the center of the bony hole at different doses. (D) Representative confocal images (100 $\times$) of calcein labeling shown in the callus of drill-hole of various groups after two weeks of treatments. (E) Quantification of the mean intensity of calcein label per pixel. All values are expressed as mean $\pm$ SEM (n= 8 rats/group); *p<0.05; compared to the control group. EAF; Ethyl acetate fraction, PTH; Parathyroid hormone (1-34)

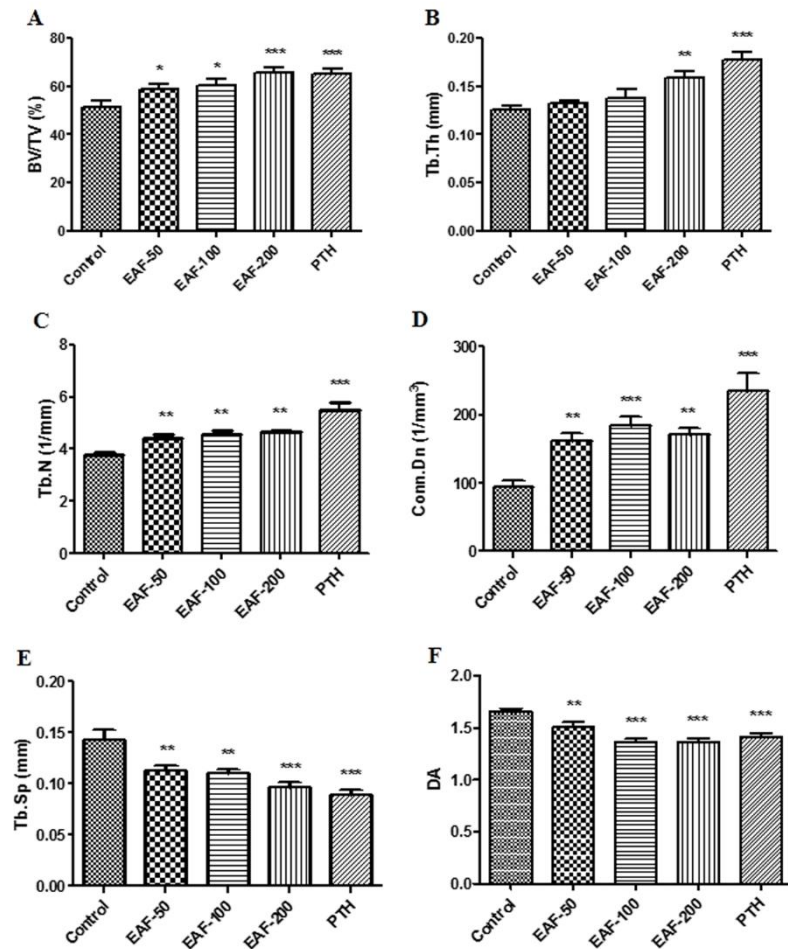


Fig 2: EAF treatment improved micro-architectural response in the drill-hole. μ -CT analysis showing BV/TV % (Bone volume/Tissue volume), Tb.Th (Trabecular Thickness), Tb.Sp (Trabecular Separation), Tb.N (Trabecular Number), DA (Degree of Anisotropy), and Conn.Dn (Connection Density). All values are expressed as mean $\pm$ SEM (n=8 rats/group); *p<0.05; **p<0.01; and ***p<0.001, compared to the control group. **EAF**; Ethyl acetate fraction, **PTH**; Parathyroid hormone (1-34).

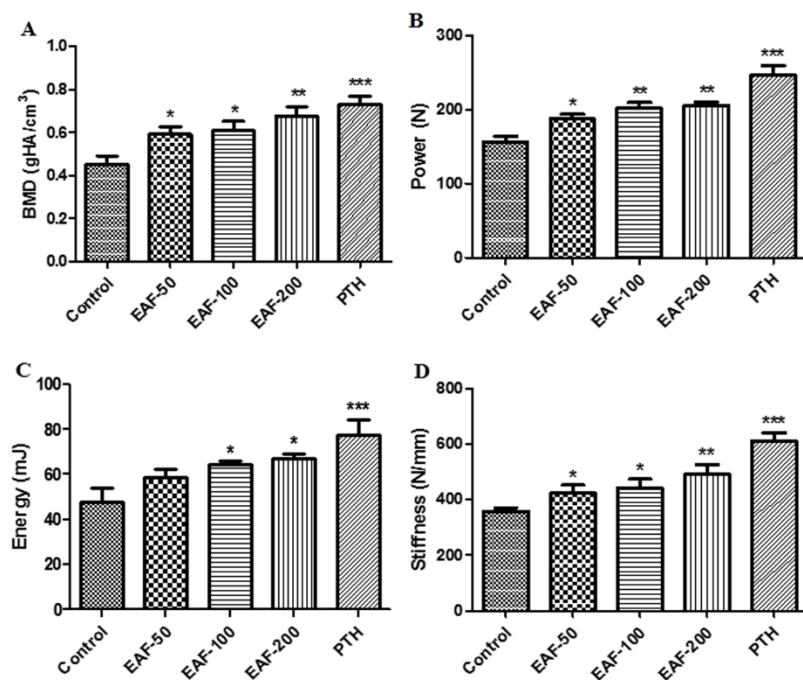


Fig 3: EAF treatment increased bone mineral density and bone strength at the fracture site. (A) vBMD, (B) Power, (C) Energy, (D) Stiffness. All values are expressed as mean $\pm$ SEM (n=8 rats/group); *p<0.05; **p<0.01; and ***p<0.001, compared to the control group. EAF; Ethyl acetate fraction, PTH; Parathyroid hormone (1-34).

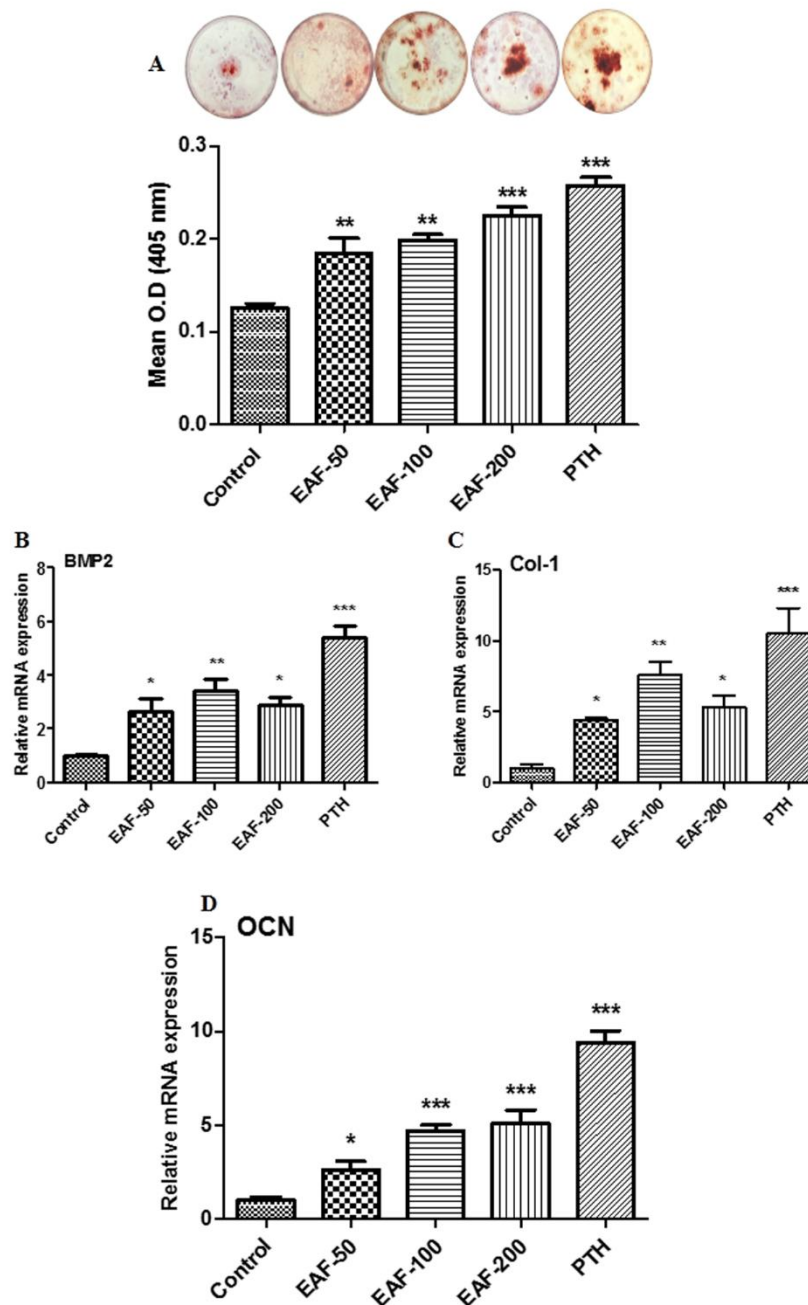


Fig 4: EAF treatment stimulated the production of osteoprogenitor cells in bone marrow cells. (A) Upper panel; Representative photomicrographs of mineralized nodules of all doses show increased intensity and larger-sized nodules by EAF treatment, compared to the control group. Lower panel; Quantification of mineralization was done by extraction of alizarin red-S dye. All values are expressed as mean $\pm$ SEM (n= 8 rats/group); **p<0.01; and ***p<0.001, compared to the control group. EAF treatment enhanced the expression of osteogenic genes in femurs of rats. qPCR determination of mRNA levels of osteogenic genes BMP-2 (B), Col-1 (C), and OCN (D) in femora of various groups was done. Each assay was performed in triplicate and results are represented as mean $\pm$ S.E.M. *p<0.05; **p<0.01; and ***p<0.001, compared to the control group. EAF; Ethyl acetate fraction, PTH; Parathyroid hormone (1-34).

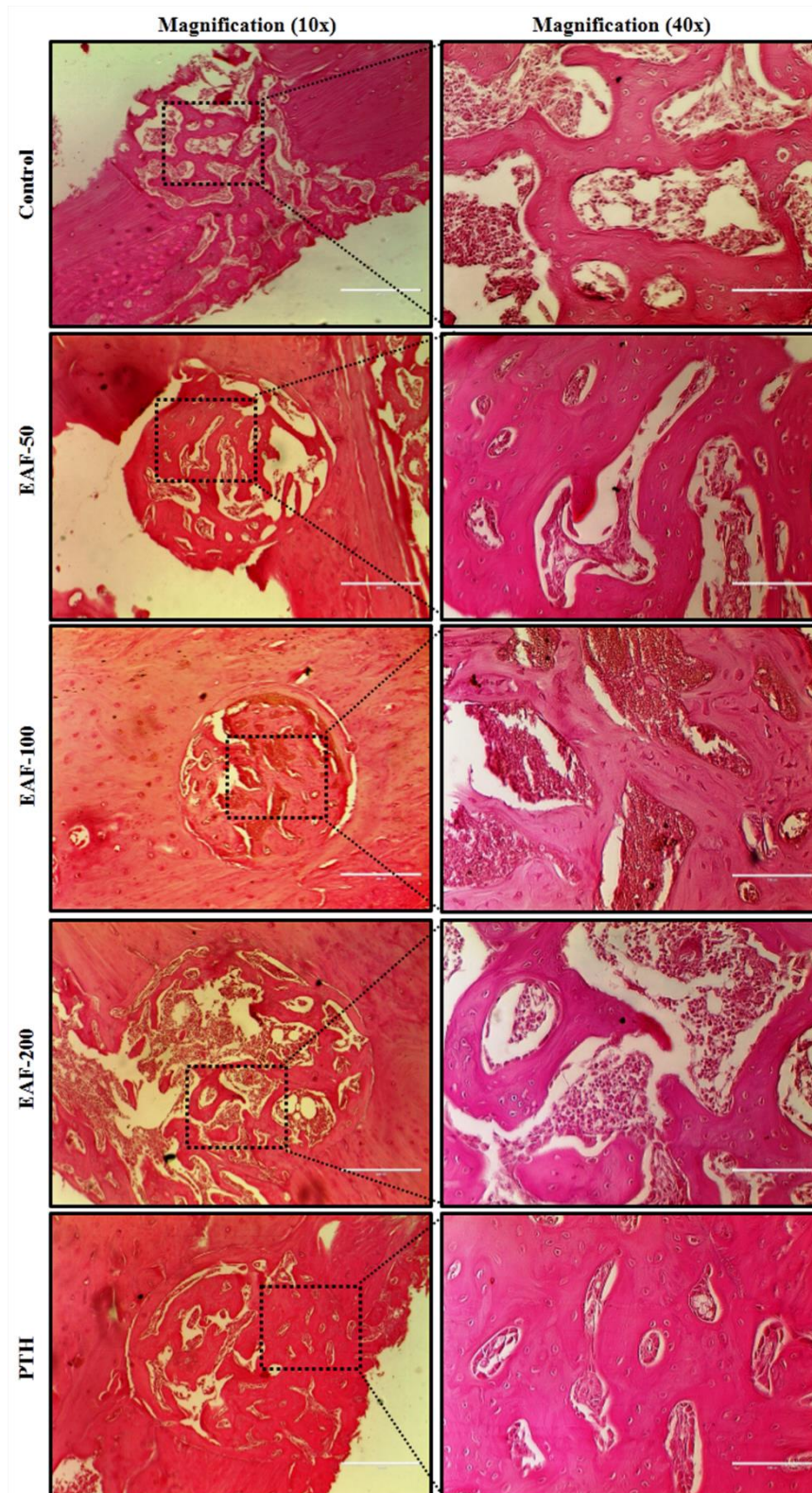


Fig 5: Bone tissue morphology was observed by H&E staining. Representative photomicrographs (10x and 40x); A larger area of regenerating bone in EAF (100 and 200 mg/kg/day) treated animals was seen by histological analysis (H&E staining) and it was same as that of PTH treated group. EAF; Ethyl acetate fraction, PTH; Parathyroid hormone (1-34).

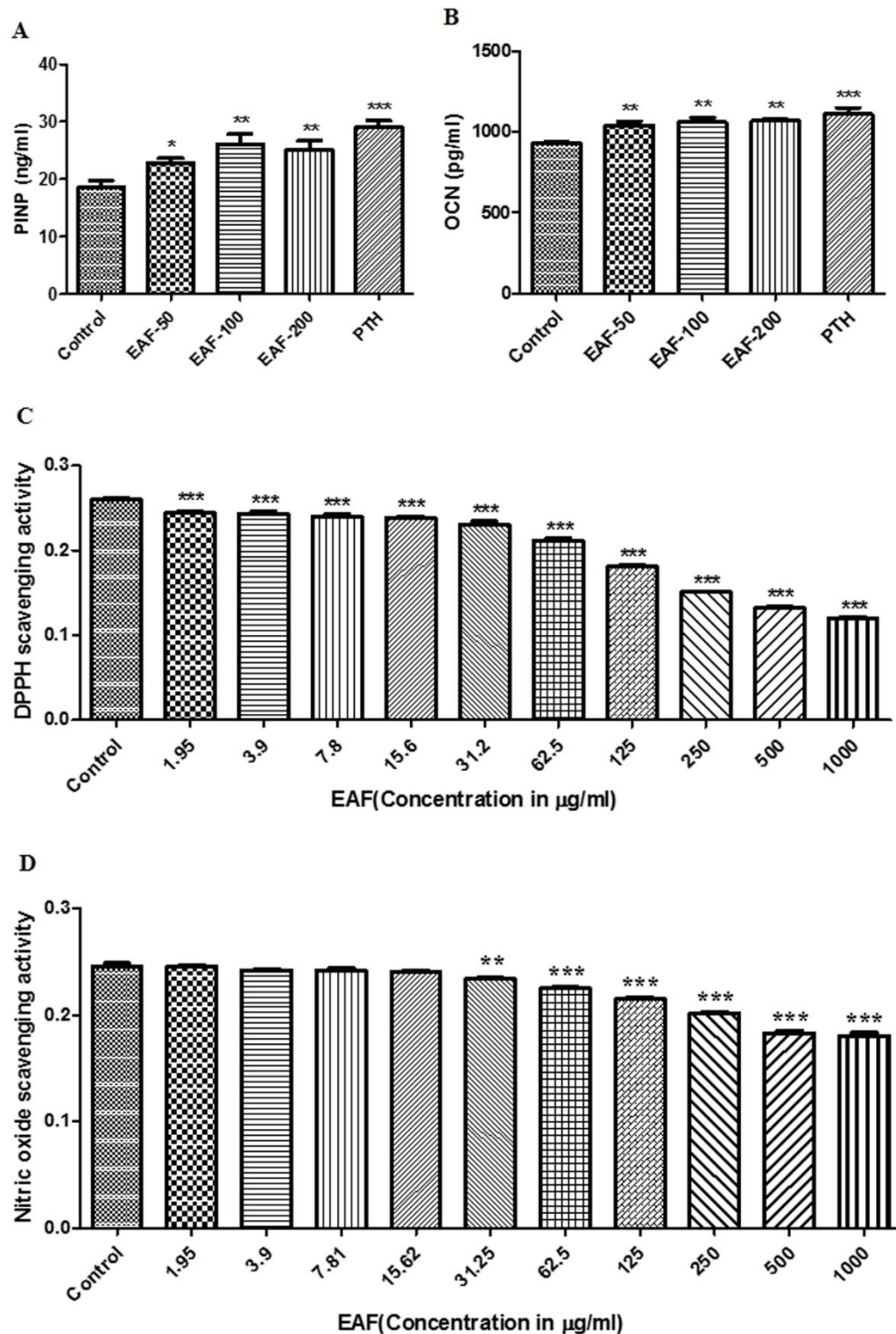


Fig 6: EAF treatment enhanced the serum levels of PINP and OCN as well as antioxidant capacity. (A and B) Serum PINP and OCN levels were significantly increased in EAF treated groups as well as in PTH group, compared to the control (vehicle treated) group. Values are expressed as Mean $\pm$ SEM; n=8 rats/group. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, compared to the control group. (C and D) DPPH and NO free radicals scavenging activity. Values are expressed as Mean $\pm$ SEM; ** $p<0.01$ and *** $p<0.001$, compared to the control. EAF; Ethyl acetate fraction, PTH; Parathyroid hormone (1-34).

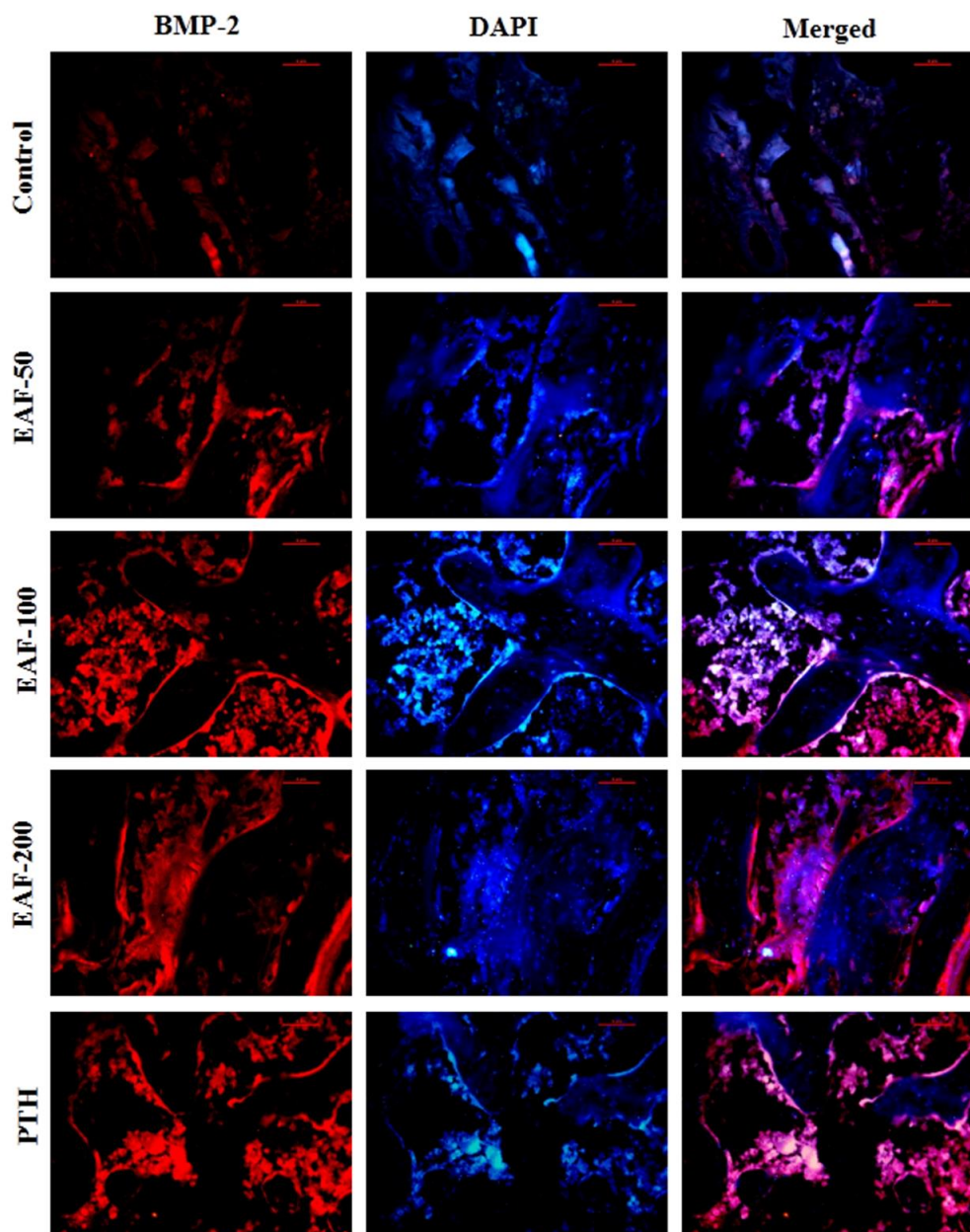


Fig 7: EAF treatment promotes the expression of BMP-2 protein in the drill-hole. Representative photomicrographs (40x); BMP-2 protein expression at the fracture site in different groups after two weeks of treatment was measured by immunofluorescence technique. Immunofluorescence staining of BMP-2 (red) was performed. Localization of the BMP-2 protein expression is demonstrated in merged images. Counterstaining was done with DAPI (blue). EAF; Ethyl acetate fraction, PTH; Parathyroid hormone (1-34).

Effect of EAF on expression of osteogenic genes in femur

Next, we assessed the effect of EAF on expression of osteogenic genes which are responsible for bone formation that included BMP-2 (bone morphogenetic protein-2), Col-1 (type1 collagen), and OCN (osteocalcin). Results from this study revealed that, daily oral administration of EAF showed an increase in the levels of mRNA expression of all three genes, compared to the control group (Fig. 4B-D). Expression of BMP-2 gene at the drill-hole site in femur bones was significantly increased by ~2.67, ~3.40, ~2.89 and ~5.39 folds with EAF (50, 100 and 200 mg/kg/day) and PTH treatment, respectively compared

to the control group. Furthermore, expression of Col-1 gene was also significantly higher by ~4.43, ~7.56, ~5.31 and ~10.48 folds as well as OCN by ~2.62, ~4.69, ~5.12 and ~9.38 folds with EAF (50, 100 and 200 mg/kg/day) and PTH treatment, respectively compared to the control group (Fig. 4B-D).

Effect of EAF on bone morphology restoration of tissue level

Oral administration of EAF promoted bone tissue morphology restoration at the drill-hole site during the progress of fracture healing. The bone tissue morphology was observed by H&E staining after EAF

and PTH treatment for two weeks. The connective tissue at the drill-hole site was replaced by the mature osteoblasts and fibrous callus [21] in the EAF (50, 100 and 200 mg/kg/day) and PTH groups, compared to the control group (Fig. 5). A larger area of regenerating bone by EAF administration at 100 and 200 mg/kg/day was seen by histological analysis and it was the same for PTH treated group. As a normal healing process, we did see callus formation in the control group as a normal healing process, however the healing in the 100 and 200 mg/kg/day groups was much better and significant than the control group (Fig. 5).

Effect of EAF on bone turnover markers

Oral administration of EAF promoted the serum levels of PINP and OCN. (Fig. 6A-B). The serum PINP levels in the EAF (50, 100 and 200 mg/kg/day) and PTH treatment groups were significantly increased by ~23.13%, ~41.11%, ~35.69% and ~56.36%, respectively compared to the control group (Fig. 6A). In addition, the serum OCN levels in the EAF (50, 100 and 200 mg/kg/day) and PTH treatment groups were significantly increased by ~11.75%, ~13.72%, ~14.66% and ~19.28%, respectively compared to the control group (Fig. 6B).

Effect of EAF on antioxidant activity

Effect of EAF on DPPH free radicals scavenging activity

We assessed the antioxidant activity of EAF at various concentrations using the DPPH assay. We observed that the violet solution of DPPH was reduced in the presence of different concentration of EAF. DPPH itself exhibits no scavenging activity (100% free radical) and therefore was used as a positive control. We assessed the scavenging activity of EAF at concentrations ranging from 1.95 µg/ml to 1000 µg/ml. We observed significant scavenging activity at all concentrations. The maximum free radical scavenging activity of EAF was ~40.94%, ~48.03%, ~52.75% at 250, 500 and 1000 µg/ml, respectively in a concentration dependent manner. Minimum scavenging activity was ~4.33% at 1.95 µg/ml (Fig. 6C).

Effect of EAF on Nitric Oxide free radicals scavenging activity

Further, we checked the effect of EAF on NO free radicals. Data revealed that maximum NO free radicals scavenging activity of EAF was ~18.06%, ~25.46% and ~26.52% at 250, 500 and 1000 µg/ml, respectively. Minimum nitric oxide inhibition was observed at lower concentration of 1.95 µg/ml and 3.9 µg/ml, respectively. EAF was effective in inhibiting the sodium nitroprusside induced nitric oxide (NO) production (Fig. 6D).

Effect of EAF on the expression of BMP-2 protein at the drill-hole site in femur

In order to confirm that EAF treatment increased the levels of BMP-2 signaling in osteoblasts cells at the drill-hole site during the progress of fracture healing after 2 weeks of treatment. With the help of immunofluorescence technique, BMP-2 localization was observed in callus (newly formed bone at fracture the site). Immunofluorescence analysis showed an intense staining for BMP-2 at the fracture site in EAF (200 mg/kg/day) and PTH treatment groups, compared to the control group [Fig. 7]. The results from this study showed that EAF promoted

the fracture healing which is regulated by activation of BMP-2 signaling pathway [21].

Discussion

Passiflora foetida, an Indian medicinal plant of the family passifloraceae is a herbaceous climber, traditionally used in asthma, eczema, chronic ulcer [13, 28]. Recently, we have reported the anti-osteoporotic activity of butanolic fraction from *Passiflora foetida* in ovariectomy-induced bone loss in mice [15]. However, there is no systematic report available to verify the ability of EAF from *Passiflora foetida* to speed up the process of fracture healing. Currently, there is no availability of orally given agent having potential to treat bone fractures. In this study, dynamic histomorphometric analysis of bone was done to check whether EAF from *Passiflora foetida* could induce the regeneration of bone at the fracture site, followed by the static histomorphometric analysis of bone by using µ-CT to check the quality of the newly formed bone (callus) at the fracture site [10, 11, 29, 30]. In addition, its osteogenic effect was also assessed in *ex-vivo* cultured BMCs. The results from this study showed that EAF from *Passiflora foetida* given orally to rats enhanced the mineral deposition at the fracture site and exhibited the improvement in quality of callus formation, which was plausible due to its stimulatory effect on osteoblast cells.

Resembling the *in vivo* bone formation at the cellular level, the EAF treatment enhanced new bone formation by increasing differentiation of the bone marrow osteoprogenitor cells cultured *ex-vivo* as well as by increasing expression of osteogenic genes in bones. BMP-2 is used in several animal models and clinical studies to enhance the process of fracture healing. Clinically, human recombinant BMP-2 is employed for the open tibial fractures to accelerate healing and reduce the need for secondary intervention by applying it directly to the fracture site as this cytokine has no oral bioavailability [31, 32]. EAF from *Passiflora foetida* significantly enhanced the BMP-2 mRNA levels as well as other osteogenic genes (OCN and Col-1) in femur bone. It seemed that the mechanism required in speeding up the process of fracture healing by the EAF involved the endogenous BMP-2 production. Furthermore, EAF treatment also enhanced the expression of BMP-2 protein at the fracture site analysed by the immunofluorescence analysis. In addition, histological analysis by H&E staining had shown a larger area of regenerating bone in EAF treated animals, and it was same as that of PTH treated group. Data taken together, suggest the potential of the EAF to promote the recruitment of osteoblast at the fracture site and preserve the quality and the integrity of the bone.

Micro-CT analysis exhibited increase in BV/TV, Tb.Th, Tb.N and Conn.Dn and decrease in Tb.Sp and DA of the callus by EAF treatment resulting in better representation of the newly formed bone over the control group. In addition, EAF from *Passiflora foetida* significantly improved vBMD at the fracture site after the oral administration for two weeks, indicating that EAF is capable of increasing the bone mass in fracture healing. Bone strength testing was also performed at the fracture site having newly regenerated bone. It was observed that EAF treatment led to increased bone strength parameters (power, energy and stiffness).

Procollagen type-I N-terminal propeptide (PINP) and the non-collagenous protein (OCN) play the important roles in bone formation. PINP provides the bone with its basic fabric and tensile biomechanical

properties and serum concentration of PINP is directly proportional to the amount of new collagen produced by osteoblasts [33, 34]. OCN maintains the normal bone mineralization, suppression of abnormal formation of hydroxyapatite crystals and the effects of cartilage mineralization [34, 35]. In this study, we found that serum PINP and OCN levels were enhanced by the treatment of EAF. All the results suggested that EAF promoted the secretion of serum bone formation markers to promote bone formation at the fracture site.

Oxidative stress is associated with the pathogenesis of bone loss leading to fractures or osteoporosis. An antioxidant has capacity to protect bone against fractures or osteoporosis via its antioxidant properties [36]. Free radicals are involved in the process of fracture healing and their higher levels may be harmful for fracture healing [37]. Osteoporosis itself may intensify oxidative stress as noticed in postmenopausal osteoporotic women, who were observed to be under oxidative stress [38, 39]. We found that EAF has anti-oxidant potential, because it was able to scavenge free radicals, which was evaluated by DPPH and NO free radical scavenging activity assays. Data show that EAF has anti-oxidant activity in a concentration dependant manner. It is surmised that EAF was able to control oxidative stress at the fracture site to generate an ideal environment for fracture healing to take place.

In folk medicines, *Passiflora foetida* is used in the form of decoction for asthma and biliousness [28]. Earlier, we have shown that butanolic fraction from *Passiflora foetida* given orally has anti-osteoporotic activity in ovariectomy-induced bone loss in mice [15]. Phytochemistry of *Passiflora* species shows the presence of alkaloids, phenols, glycosyl flavanoids and cyanogenic compounds [13]. It is believable that flavonoids and phytoestrogens found in the plant contribute to its osteoblast stimulating effect and resultant faster fracture healing process in-vivo. Overall, our results suggest that EAF from *Passiflora foetida* stimulates fracture healing and improves callus quality by enhancing the production of BMCs, followed by their differentiation to osteogenic lineage cells as a result increasing the recruitment of osteoblast cells to the fracture site as well as improving the ability of the cells to increase the production of osteogenic cytokine, BMP-2 and deposition of matrix protein, col1, eventually speed up the process of fracture healing.

Conclusion

In a preclinical set up, this study certainly showed that daily oral administration of EAF from *Passiflora foetida* stimulates the process of fracture healing. The effect of accelerating the process of fracture healing at the injury site appears to be due to the osteogenic effect of the EAF as a result stimulating the recruitment and differentiation of osteoblast cells at the injury site. This study rationalizes the traditional use of the plant in folk medicines, therefore could be taken as an alternative therapy for fracture healing. Further studies will be needed to determine the bio-active markers and their mechanism of action of the EAF from *Passiflora foetida*.

Conflict of interest

The authors declare no conflict of interests.

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